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Experiments completed in the past 6 months (Oct. 1, 1998 - March 30, 1999) were designed to characterize the localization at light and electron microscopic level of the Vascular Endothelial Growth Factor receptor Flk-1 on normal and breast cancer tissues. A novel polyclonal antibody (72 Ab) has been raised against Flk-1, affinity purified and used for these studies. Our findings show that the 72 Ab, while useful for detection by immunofluorescence of the receptor in cultured cells overexpressing Flk -1, did not give a consistent signal on samples from murine kidney and breast tumors grown on nude mice.

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George Parlade

PI - Signature Date

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(5)INTRODUCTION

Tumor growth and metastatic spread of the primary solid tumors are generally accepted to be angiogenesis dependent. The ingrowth of the surrounding vasculature to supply the tumor with nutrients and provide a conduit in which tumor cells can travel to distant sites is absolutely necessary for tumor growth beyond 2 mm in diameter and for the development of distant metastases (11-13, 17, 21).

The mechanisms by which the host vasculature invades tumors are numerous and complicated. However, the primary and critical event is the release of an angiogenic factor from the tumor cells and the surrounding extracellular matrix(14, 15) Many angiogenic growth factors have been characterized, but only one known factor, vascular endothelial growth factor (VEGF, a.k.a. vascular permeability factor, VPF) (10, 27) acceptably satisfies the many criteria to be considered an angiogenic factor outside experimental models. VEGF is angiogenic both *in vitro* and *in vivo*. VEGF is a homodimeric protein of ~46 kD produced by many cell types, including a variety of tumors, folliculostellate cells, macrophages, and possibly podocytes or capsular epithelial cells in the real glomeruli among others(9). VEGF is highly conserved with 88-98% homology among murine, rat, bovine and human sequences (5, 19, 29) and the VEGF mRNA is alternatively spliced into 4 isoforms (120, 164, 188, 204 aa in mice). There are two high affinity receptors identified for VEGF, fms-like tyrosine kinase (Flt-1, homodimer of 180 kD)(7) and fetal liver kinase (Flk-1, homodimer of 205 kD) (26). Both receptors are tyrosine kinases and appear to be exclusively expressed on endothelial cells. Although the receptors are related to the PDGF receptor, they represent a new class of receptor tyrosine kinases since their extracellular domain contains 7 immunoglobulin type repeats rather the standard 5 (26). The VEGF receptor mRNA has been detected in the endothelium of many tissues at different levels of expression, with proliferating vascular endothelial cells (due to either pathology or normal development) expressing the highest levels (23, 24). Except for some fenestrated endothelium in kidney glomerulus and choroid plexus, non-proliferating vascular

endothelium does not express detectable levels of either receptor (23). The receptors are the first proteins expressed on endothelium in development and are critically necessary for the animal development as evidenced by the fact that the receptor knock-outs are embryonic lethal (16, 28). However, no studies have adequately quantitated the levels of the receptor expression in tumor vasculature. Differences in function and *in vivo* distribution between the two receptors in normal or cancer tissues are unknown. Both the growth factor and the receptors have been shown to be intimately involved in developmental vasculogenesis (3) and angiogenesis during wound healing (22) and numerous pathologies which include diabetic retinopathy (1), reumathoid arthritis (20), chronic inflammation (30), psoriasis (8) as well as numerous malignancies (2, 4, 6, 24), including breast cancer (4, 18). In every case where angiogenesis is a prevalent pathological characteristic, VEGF has been found.

The VEGF receptors have been localized at the mRNA level by in situ hybridization. Due to the fact that commercially available antibodies are not useful reagents for immunohistochemical studies we have undertaken the task of raising antibodies which could be used in such studies. We were partially successful in the sense that we succeeded to generate an anti-Flk-1 antibody which is useful in western blotting, immunoprecipitation and apparently in immunofluorescence studies on cultured cells which overexpress the receptors.

(6)BODY OF PROPOSAL

METHODS:

Antibody production:

The C-terminal of murine Flk-1 (aa 1289-1419) and Flt-1 (aa 1277-1396) was expressed both as GST fusion protein and His tagged protein. The GST fusion protein was used as antigen for antibody production in chickens while the His tagged proteins were used for affinity purification of the antibodies.

Antibody purification from egg yolks:

Egg yolks, devoid of the amniotic sack, were diluted 4x in PBS and brought to 3.5% polyethylene glycol (PEG). The precipitated lipoproteins were centrifuged for 25 min at 5,000xg in a JA-14 rotor and the supernatant was saved. The pellet was redissolved in 2x initial yolk volume of PBS and a second round of 3.5% PEG precipitation was performed as before. The two supernatants were pooled, filtered through four layers of cheese-cloth and centrifuged for an additional 25 min at 7,500xg. The supernatant was mixed with 0.085 x volume of supernatant g PEG to precipitate IgY. The precipitate was collected by centrifugation at 5000xg for 25 min. The pellet was solubilized in PBS (2.5x initial yolk volume) and IgY was again precipitated using 3.5% PEG. The precipitate was collected by 5000xg centrifugation for 25min and redissolved in PBS (0.25x initial yolk volume). This IgY solution was chilled on ice for 10 min and mixed with an equal volume of 50% ethanol which had been chilled at -20°C. The precipitated IgY was collected by 30min centrifugation at 10,000xg, redissolved in PBS (0.25x initial yolk volume) and dialyzed over-night against 20-40 volumes of PBS to yield a total IgY fraction from egg yolk.

Affinity purification of anti-receptor polyclonal antibodies:

The His tagged C-terminal receptor peptide was coupled to AffiGel™10 matrix (BioRad Laboratories) following the manufacturer's instructions and packed into a column. Total IgY fraction from egg yolk was incubated with the column for 12-14h at 4°C with the

help of a peristaltic pump. The column was washed with 100 bed volumes of PBS and the bound antibodies were eluted with 10 bed volumes of 0.1M glycine, pH 2.5. Fractions (1.5ml) were collected onto 100µl of 1.5M Tris, pH 9.0 for antibody activity preservation.

Tissue isolation and fixation:

Mouse kidneys were flushed free of blood by an *in situ* perfusion (5 min, RT) of oxygenated PBS supplemented with 60 mM glucose followed by either excision or perfusion (10 min, RT) of different fixative solutions (either 0.5%, 1%, 2% or 4% paraformaldehyde or paraformaldehyde-lysine-periodate, PLP). The unfixed kidneys were embedded in OCT resin and snap frozen in liquid nitrogen. The fixed kidneys were excised, cut in 1mm slices and further incubated (1 h, RT) in fresh fixative (same concentration as for perfusion). The kidney slices were cut in 3x3 mm blocks (for thick sections and immunodiffusion) or 1 x 1 mm blocks for semithin cryosections and further incubated (either 5h, RT or overnight at 4°C) with several fresh fixative changes.

Breast cancer tumors grown on nude mice were harvested, embedded in OCT resin and snap frozen in liquid nitrogen and stored at -80°C until further examination.

Immunofluorescence on thick sections

Thick sections (~5µm) were cut from kidney blocks. In the case of unfixed kidneys the sections were fixed on slides (20 min, RT) using different fixatives (acetone/methanol, 70% ethanol, 2% paraformaldehyde or PLP). The kidney sections from both *in situ* and "on slide" fixed specimens were quenched (30 min, RT) in different blocking solution (either 5% fetal calf serum, 1% cold water fish skin gelatin or 1% bovine serum albumin each supplemented with 0.01M glycine in PBS), incubated (1-16h) with the anti-Flk-1 antibody (72 Ab) diluted (1:10-1:200) in the appropriate blocking solution, washed (3 x 15 min, RT) in blocking solution and incubated (1-4 h, RT) with the reporter antibody (rabbit anti chicken IgY- FITC), washed again as above, mounted and finally examined under an Axiophot microscope using appropriate filters.

In the case of the breast cancer tumors, the procedures were carried on similarly as in the case of the mouse kidneys, except that the tumors were not perfused but trimmed in small blocks and fixed by diffusion using the same panel of fixative solutions.

Immunofluorescence on semithin frozen sections:

Small blocks (1x1 mm) from fixed kidneys were cryoprotected by infiltration (12-16h at 4°C) with a solution containing 1.5M sucrose, 50% polyvinylpyrolidone in PBS and stored frozen in liquid nitrogen. Semithin frozen sections (0.5-1μm) were sectioned on a Reichert ultramicrotome equipped with a F4 cryoattachment, transferred to slides and stained and examined as in the case of the thick sections. This procedure has the advantage of increased resolution over the thick sections.

Immunodifusion:

Preembedding immunocytochemistry was performed as previously described in Predescu et al. (25). Small mouse kidney blocks (~3 x 3 mm) were cryoprotected by infiltration (12-16h at 4°C) with a solution containing 1.5M sucrose, 50% polyvinylpyrolidone in PBS and stored frozen in liquid nitrogen. Thick cryostat sections (~45μm) cut from the fixed blocks were rinsed (5 x 5 min) and incubated for overnight at 4°C in 10% goat serum in PBS, quenched in 1% BSA in PBS (PBSA) for 30 min at RT and incubated with the anti-Flk-1 pAb diluted (1:30-1:50) in PBSA. The sections were washed 3 x 30min in PBSA at RT, incubated with the rabbit anti-chicken IgY 5nm gold conjugated antibody (1:100 dilution in PBSA) for 12-16h at 4°C. After final washes as above the antibody-antigen complexes were stabilized by fixation (1h at RT) in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed in 1% OsO4 in acetate veronal buffer, pH 6.8 (1h on ice), stained in the dark (1h at RT) with Kellenberger uranyl acetate, dehydrated through graded ethanol and finally embedded in Epon 812 resin. Tissue blocks were cured for 48h at 90°C, and ~50nm sections, cut on a Reichert microtome, mounted on formvar coated nickel grids were stained with 2% uranyl acetate and saturated

lead citrate. The stained sections were examined and photographed in a Phillips CM10 electron microscope.

This approach for immunolabeling relying on the diffusion of the antibodies along vascular and perivascular spaces provides adequate sampling of morphologically well preserved vascular endothelia.

RESULTS AND DISCUSSION:

Antibody validation:

ELISA: Affinity purified anti-Flk-1 or anti-Flt-1 were checked for specificity and activity in an ELISA assay using serial dilutions of the antibody on either 100ng C-terminal His tagged Flk-1 or Flt-1 peptide/plate well, respectively. The bound anti-receptor antibody was detected using an anti- chicken IgY HRP conjugated reporter antibody and TMB substrate for colorimetric reaction. Negative controls represented by either preimmune IgY or irrelevant His tagged peptide were used.

Both anti-Flk-1 and anti-Flt-1 recognized with high affinity the respective His tagged peptides. To further test the antibodies we coated ELISA plates with membrane or cytosolic proteins from MS1 endothelial cells grown in culture. Only Flk-1 recognized the membranes from MS1 cells.

Immunoblotting: MS1 endothelial cells (expressing high-levels of receptors) were separated into a total membrane and cytosol fraction. The proteins from each fraction were resolved by SDS-PAGE, transferred to a PVDF membrane and strips containing the membrane proteins on one lane and cytosol protein on another lane were cut. The strips were immunoblotted with serial dilutions of either anti-Flk-1 or anti-Flt-1 antibody. Positive and negative controls represented by commercially available anti-Flk-1 pAb (Santa Cruz Laboratories) and preimmune IgY, respectively, were used. Only the Flk-1 antibody (to which we will refer as the 72 Ab) recognized a band at ~205 kD in the MS1 total membrane fraction with higher affinity that the commercial antibody. No band was detected in the

cytosol. These results suggest that the 72 Ab recognizes specifically Flk-1. The Flt-1 antibody did not recognize any band even though we tried it repeatedly on different amounts of protein/lane, different blocking conditions (5% nonfat milk, 5% calf serum, 5% fetal calf serum, 2% gelatin, 4% BSA).

Both antibodies were also tried on breast cancer tumors lysates. Unfortunately, the blots came out not clean enough (in spite of our repeated attempts in changing the blotting conditions) to allow conclusive identification of the receptors.

Immunoprecipitations: MS1 endothelial cells membranes were extracted for 1 h on ice in immunoprecipitation buffer (20mM Tris, pH 7.5 containing 1% NP40, 0.4% deoxycholate, 0.1% SDS, 150mM NaCl, 1mM EDTA, 1 mM PMSF and protease inhibitors cocktail). The extract was clarified by centrifugation and the extracted proteins (200-600 μg) were incubated by gentle agitation with 10μg of anti-receptor pAb for 14 h at 4°C. This mixture was further incubated for 4 h at 4°C with anti-chicken IgY antibodies insolubilized onto agarose beads to precipitate the antigen-antibody complexes. The beads were collected by centrifugation, washed and boiled in SDS-PAGE sample buffer and collected by centrifugation. The supernatant containing the solubilized antigen-antibody complexes was resolved by 6 % SDS-PAGE, transferred to PVDF membrane and immunoblotted using the commercially available antibodies.

Only the anti-Flk-1 pAb precipitated a band of ~205 kD (as judged by silver staining), band which was recognized by the commercially available antibody which suggests that this antibody recognizes Flk-1 in immunoprecipitation.

Immufluorescence on MS1 cells

MS1 cells were stained with either the anti-Flk-1 pAb (72 Ab) or anti-Flt-1 pAb followed by FITC or rhodamine reporter antibodies. Only the 72 Ab gave a punctate staining on these cells, signal which appeared to be specific as it was not detected in the negative controls represented by either replacing of the primary antibody with preimmune IgY or omitting it. The only fixation method which preserved the antigen was

Acetone/Methanol fixative. No signal was detected when the cells were fixed in paraformaldehyde 1-4%, PLP or 75% Ethanol. These results suggest that the 72 Ab could be useful in immunohystochemical studies but the antigen which is recognized by this antibody is fixation sensitive.

The anti-Flt-1 pAb gave no signal in any of the fixation methods we used.

Immufluorescence and Immunogold labeling:

In order to optimize the staining procedure for the anti-Flk-1 antibody we used mouse kidney specimens prepared in different conditions as Flk-1 has been documented to be expressed on the endothelial cells in the normal kidney glomerulus. We examined these preparations at different levels of resolution (thick and semithin frozen sections by immunofluorescence and ultrathin sections by immunodifusion and electron microscopy).

Unfortunately, we were not able to find the conditions in which the 72 Ab would give us a consistent and reliable staining of the glomerulus by immunofluorescence even though we repeatedly used different conditions (see Methods). We also went to the electron microscopic level in order to see if there is any staining at all and which is not detectable by immunofluorescence. In the case of the immunodifusion studies the staining was extremely weak at levels comparable with the control experiments represented by either replacement of the primary antibody with preimmune IgY or omission of the primary antibody.

Even though the expression of the receptor on kidney glomerulus is at lower levels that those envisioned on the endothelial cells from the tumor vessels we felt that the anti-Flk-1 72 Ab was not an appropriate reagent for immunolocalization of this receptor. However, we attempted to use the 72 Ab to stain "on slide" fixed thick sections of snap frozen breast cancer tumors but no specific signal was detected.

(7)KEY RESEARCH ACCOMPLISHMENTS

•Prepared antibodies against the VEGF receptors, Flk-1 and Flt-1.

(8) REPORTABLE OUTCOMES : None

(9)CONCLUSIONS

In order to complete the Task 4 of the grant proposal, represented by specific localization of the VEGF receptors (contingent upon generation of sufficiently sensitive reagents), we have generated antibodies against the C-terminus of either Flk-1 or Flt-1. The chicken was chosen for antibody production because it is a documented suitable host for generating antibodies against proteins which are highly conserved in mammals and our past repeated attempts to raise antibodies to peptides or fusion proteins derived from VEGF receptors in rabbits have failed. We were somewhat successful in the respect that only the anti-Flk-1 antibody (72 Ab) recognized the receptor in ELISA, immunoblotting or immunoprecipitation assays. The antibody against Flt-1 while recognizing the antigen in ELISA assays is not an useful reagent for any of the other procedures.

The immunolocalization studies carried on under Task 4 provisions have not led to any conclusive results in spite of a considerable effort. The anti-Flk-1 antibody while apparently useful for immunofluorescence studies on cells grown in culture and overexpressing the receptors, gave only negative results while used on normal or tumor tissues.

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